Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain

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Published online 10 March 2003; doi:10.1038/nm837

The subcortical white matter of the adult human brain harbors a pool of glial progenitor cells. These cells can be isolated by fluorescence-activated cell sorting (FACS) after either transfection with green fluorescent protein (GFP) under the control of the *CNP2* promoter, or A2B5-targeted immunotagging. Although these cells give rise largely to oligodendrocytes, in low-density culture we observed that some also generated neurons. We thus asked whether these nominally glial progenitors might include multipotential progenitor cells capable of neurogenesis. We found that adult human white-matter progenitor cells (WMPCs) could be passaged as neurospheres *in vitro* and that these cells generated functionally competent neurons and glia both *in vitro* and after xenograft to the fetal rat brain. WMPCs were able to produce neurons after their initial isolation and did not require *in vitro* expansion or reprogramming to do so. These experiments indicate that an abundant pool of mitotically competent neurogenic progenitor cells resides in the adult human white matter.

The adult human subcortical white matter harbors a population of mitotically competent glial progenitors that comprise as many as 3% of its cells[1,2]. These cells may be extracted from brain tissue using FACS after transfection with GFP-encoding plasmids driven by the promoter for *CNP*, an early oligodendrocytic transcript[2,3]. The cells express the immature neural ganglioside recognized by monoclonal antibody A2B5 but do not express more mature markers of glial lineage. We previously noted that when grown at high density, pCNP2:hGFP$^+$ progenitors gave rise to glia, largely oligodendrocytes. Nonetheless, in low-density culture after high-purity FACS, pCNP2:hGFP$^+$ cells often generated βIII-tubulin$^+$ neurons[2]. Because neurogenesis was never observed from pCNP2:hGFP$^+$ cells in higher-density or unsorted cultures, we postulated that the restriction of these progenitor cells to the oligodendroglial phenotype might be an effect of environmental cues rather than a function of autonomous commitment. Once isolated into high-purity, low-density culture, and therefore removed from any paracrine or autocrine influences, human subcortical pCNP2:hGFP$^+$ cells were able to generate neurons as well as glia[2]. It was subsequently reported[4] that glial progenitors from the postnatal rat optic nerve could also generate neurons after serum- or bone morphogenetic protein–induced phenotypic instruction and basic fibroblast growth factor (bFGF)–stimulated expansion. Similar work showed that progenitor cells of the adult rat forebrain parenchyma could also generate neurons after prolonged *in vitro* expansion in bFGF[5]. Taken together, these findings indicated that glial progenitor cells might retain substantial phenotypic plasticity.

We asked whether some fraction of the nominally glial progenitors of the adult human subcortical white matter might actually be parenchymal neural stem cells. Specifically, we asked whether single, sorted WMPCs could generate multiple neural phenotypes, and if so, whether they were capable of expansion and self-renewal. In addition, we investigated whether this process requires de-differentiative reprogramming to an intermediate phenotype, or whether simply removing these cells from their local environment and mitotically expanding them in bFGF might suffice to permit these cells to act as multipotential progenitors. In doing so, we tested the hypothesis that the phenotypic plasticity of adult WMPCs might be tonically restricted by the adult parenchymal environment, rather than irreversibly lost with development.

WMPCs were isolated by *CNP*- and A2B5-based sorting

White matter was dissected from surgical samples taken at the time of temporal lobectomy for epilepsy, aneurysm, and post-traumatic decompression ($n = 21$). The tissues were dissected free of adjacent cortex and ventricular epithelium, and enzymatically dissociated to single-cell suspension as described[2]. The dissociates were plated onto laminin (100 μg/ml) in DMEM/F12/N1 supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and platelet-derived growth factor (PDGF)-AA (20 ng/ml). To identify oligodendrocyte progenitors, the dissociates were trans-

fected with pCNP2:hGFP, the transcription of which results in GFP expression by oligodendrocyte progenitor cells[2].

To avoid both the temporal lag between transfection and GFP expression and the inefficiency of plasmid transfection, cultures were also sorted on the basis of A2B5 surface immunoreactivity, which can serve as a surrogate marker for pCNP2:hGFP$^+$ WMPCs *in vitro*[2]. Immunostaining showed that 84 ± 8.3% of pCNP2:GFP$^+$ cells expressed A2B5 (ref. 2). GFP-based FACS gated 0.49 ± 0.15% of all white-matter cells as pCNP2:hGFP$^+$ (mean ± s.e.m.; $n = 3$ patients; Fig. 1*a*). Matched cultures transfected with pCMV:GFP had a net transfection efficiency of 13.1%. Thus, the predicted incidence of pCNP2:hGFP$^+$ cells in the white matter was 3.7% (= 1 + 0.131 × 0.49), consistent with our prior estimates of the incidence of this phenotype[2]. From the same samples, A2B5-based FACS gated an average of 3.1 ± 0.7% ($n = 3$) of the white-matter cell population (Fig. 1*b*). The greater than six-fold increase in net yield when A2B5 was used (3.1% versus 0.49%) reflected the higher efficiency of A2B5 immunodetection relative to pCNP2:hGFP plasmid transfection. On this basis, we used immunomagnetic sorting (IMS) to select A2B5$^+$ cells from adult white-matter dissociates. By IMS, the incidence of A2B5-sorted cells in white matter dissociates was 3.6 ± 0.3% ($n = 21$) with a median of 3.1%. This improved yield was accomplished with no appreciable loss of cell-type specificity, in that the A2B5$^+$ cells overlapped entirely with the sort profiles of pCNP2:hGFP$^+$ cells and each isolate generated O4$^+$ oligodendrocytes with similar efficiency (Fig. 1*c–f*). Thus, A2B5-based FACS and IMS identified WMPCs homologous to those recognized by pCNP2:GFP-based FACS, while permitting higher-yield isolation of these cells.

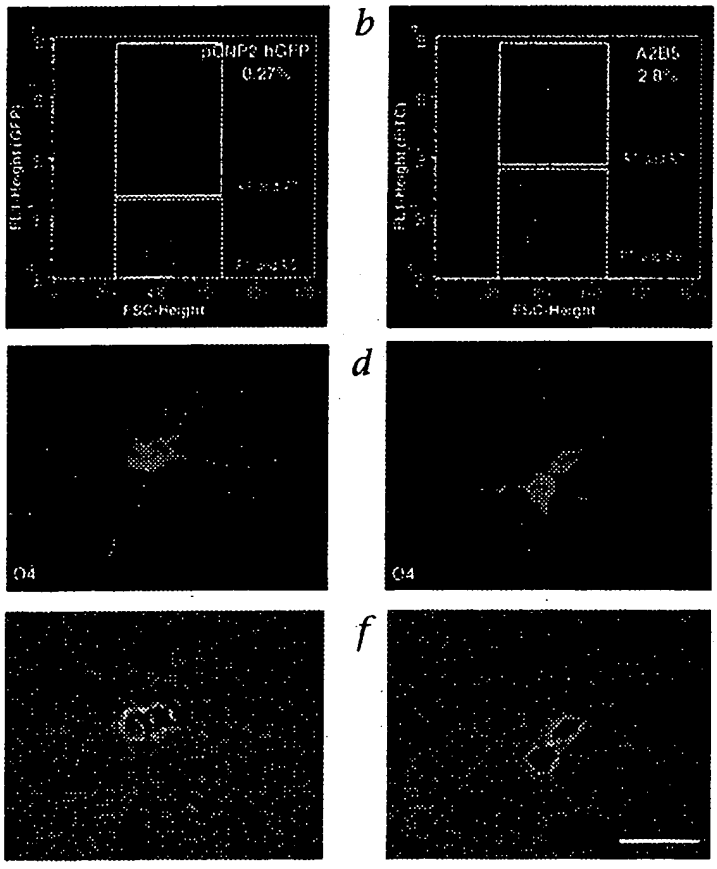

Fig. 1 A2B5-based FACS selects oligodendrocyte progenitor cells. *a* and *b*, FACS graphs showing the extraction of pCNP2:hGFP$^+$ (*a*) and A2B5$^+$ (*b*) WMPCs from an adult human white-matter dissociate. Forward scatter (FCS), a measure of cell size, is plotted against fluorescence intensity (Fl-1). When pCNP2:hGFP- and A2B5-based sorts were directly compared, their plots showed overlapping profiles, but A2B5$^+$ cells were >6-fold more abundant than their pCNP2:hGFP$^+$ counterparts, reflecting the higher efficiency of A2B5 surface tagging. *c–f*, Progenitors sorted by pCNP2:hGFP (*c* and *e*) and A2B5 (*d* and *f*) gave rise to O4$^+$ oligodendrocytes. A2B5-based surface antigen sorting may thus be used as a higher-yield alternative to pCNP2:hGFP transfection–based FACS for isolating WMPCs. Scale bar, 24 μm.

Adult WMPCs gave rise to multipotent neurospheres

To assess the expansion capacity of pCNP2:hGFP- and A2B5-sorted cells, we propagated sorted isolates of each in suspension[6–8]. The cells were distributed into 24-well plates at 50,000 cells per 0.5 ml in serum-free media (SFM) supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and PDGF-AA (20 ng/ml), a combination that permits the expansion of human WMPCs[2]. Seven days later, the cells were switched to SFM with bFGF alone (20 ng/ml)[6]. Over the next 10 d, neurospheres—spherical masses of cells that expand from single parental progenitors—arose in these cultures, such that by 3 weeks after sorting, there were 84.8 ± 9.0 spheres/well (n = 4 patients). These neurospheres were typically >150 μm in diameter and included 46.5 ± 8.2 cells/sphere (Fig. 2*a* and *b*). Thus, single WMPCs of the adult human brain were capable of generating neurospheres.

To establish the lineage potential of single adult human WMPCs, we dissociated the resultant primary neurospheres and passaged them into new wells. Alternatively, some were plated onto substrate to permit their differentiation. Immunostaining showed that both pCNP2:hGFP$^+$ and A2B5$^+$ progenitor–derived spheres gave rise to all major neural phenotypes (Fig. 2*d* and *e*). Among those cells passaged from primary spheres, secondary spheres were observed to arise within two weeks after passage. After expansion, these secondary spheres were similarly plated on substrate, raised for one to two weeks and fixed. Immunolabeling confirmed that virtually all secondary spheres generated both neurons and glia together (Fig. 2*c* and *e*). In addition, when the mitotic marker BrdU was added to A2B5-sorted cells, BrdU-incorporating neurons, oligodendrocytes and astrocytes all emerged from the spheres generated (Fig. 2*f–i*). The persistence of mitotic neurogenesis and gliogenesis by single spheres indicated that they contained cycling multipotential cells. The secondary spheres were probably of clonal origin, given the low plating density of the single cells from which each was derived and the fact that the sphere-forming cells originated from primary spheres that had themselves expanded from single-cell dissociates. These data indicate that single progenitor cells of the adult human white matter are both clonogenic and multipotent.

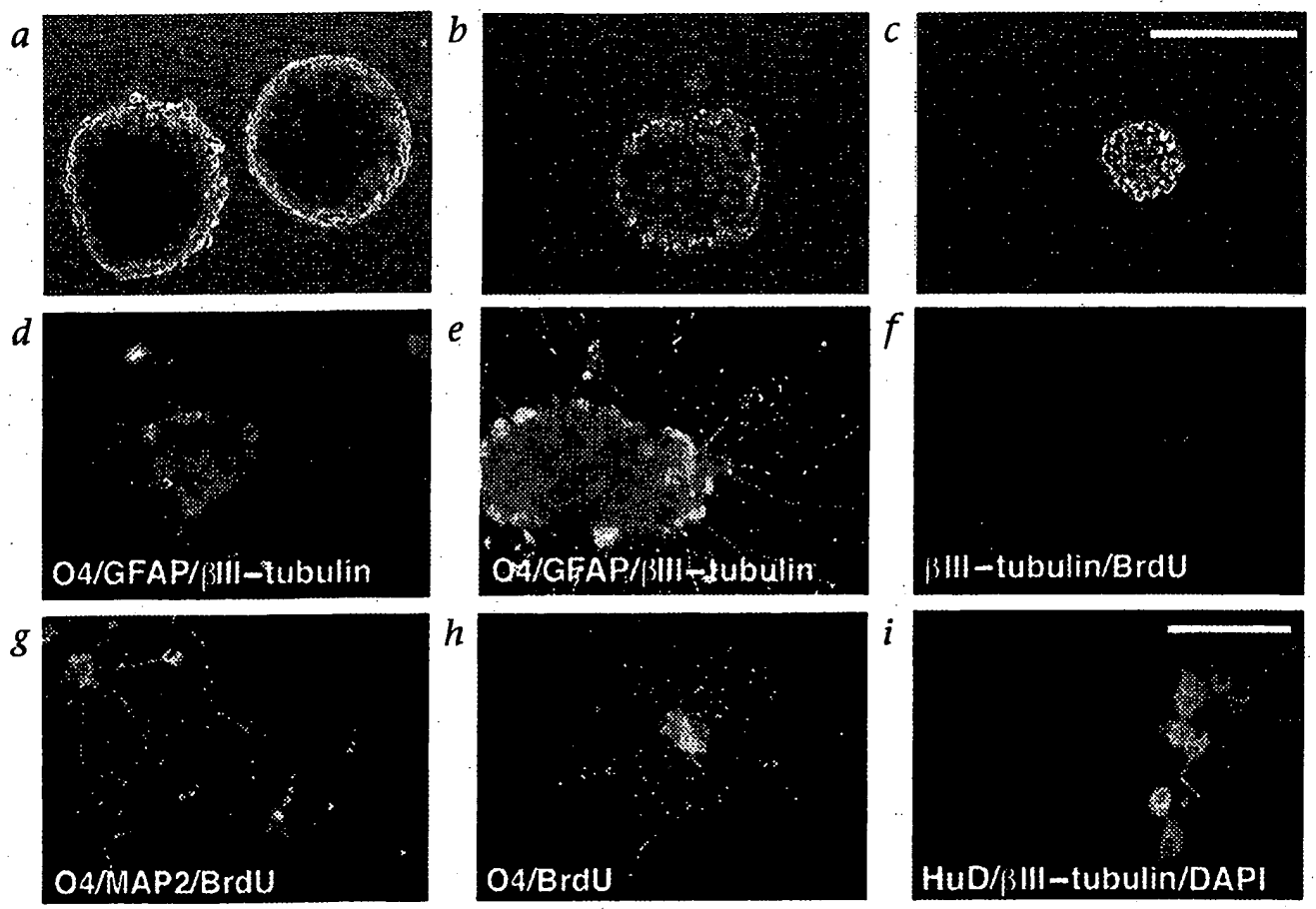

Fig. 2 Adult human WMPCs give rise to multipotential neurospheres. ***a***, First-passage spheres generated from A2B5-sorted cells 2 weeks after sorting. ***b***, First-passage spheres arising from pCNP2:hGFP-sorted cells, at 2 weeks. ***c***, Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. ***d***, Once plated onto substrate, the primary spheres differentiated into βIII-tubulin$^+$ neurons (red), GFAP$^+$ astrocytes (blue) and O4$^+$ oligodendrocytes (green). ***e***, Neurons (red), astrocytes (blue) and oligodendrocytes (green) arose similarly from spheres derived from pCNP2:GFP-sorted WMPCs. ***f–h***, BrdU incorporation (blue) showed that new neurons (*f*, βIII-tubulin (red); *g*, MAP2 (red)) and oligodendrocytes (*h*, O4 (green)) were generated *in vitro*. ***i***, βIII-tubulin$^+$ neurons (green) co-expressed neuronal Hu protein[40,41] (red), yielding double labeling (yellow). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm (*a–e*) or 40 μm (*f–i*).

Single WMPCs remained multipotential with passage

The serial propagability of sorted WMPCs from neurospheres in low-density dissociates suggested the clonal derivation of each individual sphere[9–11]. To further validate the clonal origin of neurons and glia arising within single spheres, we used lentiviral GFP to genetically tag and follow single WMPCs. A2B5$^+$ cells were tagged, 2–5 d after sorting, with a lentivirus expressing GFP under cytomegalovirus (CMV) promoter control[12–14]. At 10 PFU/cell, 23% of the cells expressed GFP by one week after sorting, yielding a mixture of GFP$^-$, GFP$^+$ and mixed spheres in the resultant cultures (Fig. 3*a–b*). These primary spheres were triturated two to four weeks later to single-cell suspensions and passaged into bFGF at ~3,000 cells/well. Under these conditions, 40.8 ± 12.9 secondary spheres/well were generated, indicating a clonogenic cell incidence of 1.3% (n = 5). Of these secondary spheres, 47.2 ± 10.8% contained only GFP$^+$ cells (Fig. 3*c–d*) whereas 30.9 ± 6.9% harbored no GFP$^+$ cells. The relative uniformity of GFP expression, or lack thereof, among the cells within a given sphere indicated that most spheres were clonally derived ($P < 0.005$ by χ^2 analysis). This tested the null hypothesis that the spheres arose from non-clonal aggregation of two or more cells, each of which was equally likely to be GFP$^+$ or GFP$^-$. When the single spheres were plated onto polyornithine and fibronectin and their outgrowth assessed two weeks later, all gave rise to both neurons and glia (Fig. 3*e–g*). Because most secondary spheres were likely to have been clonally derived, and all included neurons as well as glia (38 of 38 spheres; n = 4 samples), single WMPCs must have given rise to neurons and glia together.

We next asked if the neurogenic capacity and multilineage competence of WMPCs were maintained with passage. Primary spheres were raised serially in bFGF/NT3/PDGF-AA for 7 d, DMEM/F12/N1 with 15% serum/PDGF-AA for 4 d, and serum-free DMEM/F12/N1 with bFGF for 10 d. Cells were then dissociated and replated in bFGF at 3,000 cells/well in a 24-well plate. Secondary spheres arose within two weeks from 1.1 ± 0.3% of these cells (n = 8). After more than two weeks of further expansion, the secondary spheres were plated on polyornithine and fibronectin and were fixed and immunostained two weeks later (seven to nine weeks after sorting). Whereas primary spheres consisted of 21.7 ± 4.3% βIII-tubulin$^+$ neurons, 17.7 ± 3.9% glial fibrillary acidic protein (GFAP)$^+$ astrocytes and 46.7 ± 5.9% O4$^+$ oligodendrocytes (n = 3), secondary spheres consisted of 16.0 ± 2.5% neurons, 19.3 ± 3.2% astro-

cytes and 46.4 ± 2.4% oligodendrocytes (n = 3). Most of the neurons were GABAergic, by virtue of their expression of glutamic acid decarboxylase-67 (GAD67) (Fig. 4*a–c*). Because the relative proportions of neurons, oligodendrocytes and astrocytes in secondary spheres were similar to those in primary spheres, we concluded that WMPCs retained multilineage competence with expansion.

WMPC-derived neurons become functionally mature

The calcium responses and membrane currents of WMPC-derived neurons were assessed to establish their ability to respond to depolarizing stimuli. Primary spheres (n = 12 fields, derived from 3 brains) were plated on fibronectin to permit neuronal outgrowth, and assessed 14 d later for their calcium responses to depolarizing stimuli. The cultures were then loaded with the calcium indicator dye Fluo-3 and serially exposed to both 100 μM glutamate and 60 mM potassium during confocal microscopy. Astrocytic responses to depolarization were minimal under these culture conditions, as previously noted. In contrast, neuron-like cells displayed rapid, reversible, >100% elevations in cytosolic calcium in response to potassium, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 4*d–f*). The neuronal phenotype of these cells was then validated by immunostaining for βIII-tubulin.

We then asked whether WMPC-derived neurons would be able to develop the fast sodium currents and action potentials characteristic of electrophysiologically competent neurons. We used whole-cell patch-clamp recording during current stimulation to assess the response of WMPC-derived neurons that arose from plated secondary spheres derived from A2B5-sorted isolates. A total of 58 WMPC-derived fiber-bearing cells were recorded, in 5 cultures derived from 3 patients. Of these, 13 showed voltage-activated sodium ion currents (I_{Na}) of >100 nA, and 7 had I_{Na} > 600, compatible with the fast sodium currents of neuronal depolarization[15,16]. Accordingly, whereas two of five cells with I_{Na} > 800 generated stimulus-evoked action potentials (Fig. 4*g–h*), none did so with I_{Na} < 800. In addition, none of 26 morphologically non-neuronal cells showed substantial (≥100 pA) current-induced sodium currents. Together, these results indicated that neurons arising from adult human WMPCs developed mature electrophysiologic functions, including both fast sodium currents and action potentials.

WMPCs generated neurons without reprogramming

Glial progenitor cells from the postnatal rat optic nerve can generate neurons, under conditions that have been described as 'reprogramming' glial progenitors to multilineage competence[4]. In that study, neurogenesis was achieved by first instructing the cells to an intermediary astrocytic lineage using either serum or bone morphogenetic protein-2, followed by bFGF-stimulated mitogenesis. We asked whether such reprogramming steps are required for the generation of neurons from adult human WMPCs, or whether simple expansion under minimal conditions *in vitro*, with the removal of these cells from their environment, might be sufficient to permit neurogenesis by these cells. Sorted A2B5$^+$ cells were cultured in several permutations of mitogenic and differentiative conditions to identify the minimal conditions permissive for lineage diversification. We compared the phenotypes generated under three conditions: (i) bFGF/NT3/PDGF-AA in SFM (composed of DMEM/F12/N1) for 7 d, followed by 15% FBS/ PDGF-

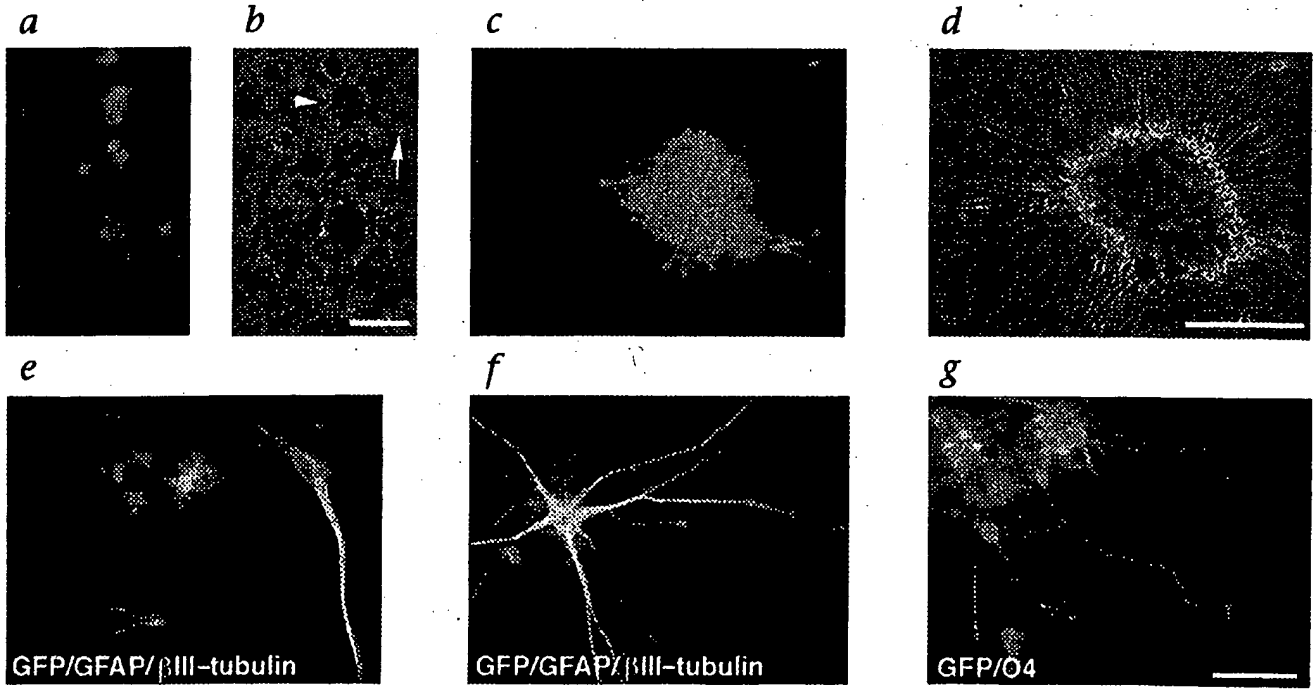

Fig. 3 Single lentiviral GFP-tagged WMPCs generated neurons and glia. A2B5-sorted WMPCs were infected with a lentivirus encoding enhanced GFP[14], 5 d after sorting. ***a*** and ***b***, Secondary spheres subsequently derived from infected cells harbored either GFP-tagged cells (arrowhead), untagged cells (arrow) or, less commonly, both. ***c*** and ***d***, GFP$^+$ secondary sphere 1 week after plating. ***e*** and ***f***, βIII-tubulin$^+$ neurons (red) and GFAP$^+$ astrocytes (blue) arising from a single clonally derived GFP$^+$ secondary sphere. ***g***, GFP$^+$ (green) and O4$^+$ (red) oligodendrocytes arising from a secondary sphere. Scale bars, 100 μm (*a* and *b*), 60 μm (*c* and *d*) or 40 μm (*e–g*).

AA for 4 d and SFM with bFGF for two weeks; (ii) bFGF/NT3/PDGF-AA in SFM continuously for three weeks; and (iii) bFGF alone in SFM for three weeks. The first condition was intended to promote initial differentiation in serum, whereas the latter two groups were designed to skip this glial differentiative step[4].

The A2B5-sorted progenitors yielded spheres under each of these conditions; however, both the number of spheres and the percentage of neurons generated by each differed as a function of treatment. Cultures maintained in base media alone or in bFGF-supplemented media had 5.9 ± 1.7% and 7.2 ± 2.1% βIII-tubulin$^+$ neurons, respectively (n = 3 patients). When matched WMPC-derived spheres were sequentially raised in bFGF/NT3/PDGF-AA with 15% serum and bFGF, 18.2 ± 2.2% of the cells were βIII-tubulin$^+$ (Fig. 5*a*). A similar proportion of neurons (22.5 ± 1.9%; n = 3) was generated by those neurospheres maintained in SFM with bFGF/NT3/PDGF-AA. Serum exposure was therefore not required for A2B5$^+$ cells to generate neurons. Indeed, no specific signals seemed necessary for neuronal instruction, besides those provided by PDGF and NT3. These data indicated that antecedent astrocytic differentiation was not a necessary prerequisite to neurogenesis by adult WMPCs. These cells required neither prolonged mitogenic expansion, nor specific dedifferentiation steps, to generate neurons as well as glia[4].

Although both PDGF and NT3 promote oligodendrocyte production by glial progenitors of the rat optic nerve[17,18], each can induce neuronal differentiation in less-committed hippocampal and ventricular zone neural progenitors[19,20]. As such, their neurogenic effects on adult WMPCs may reflect the relatively undifferentiated state of these cells.

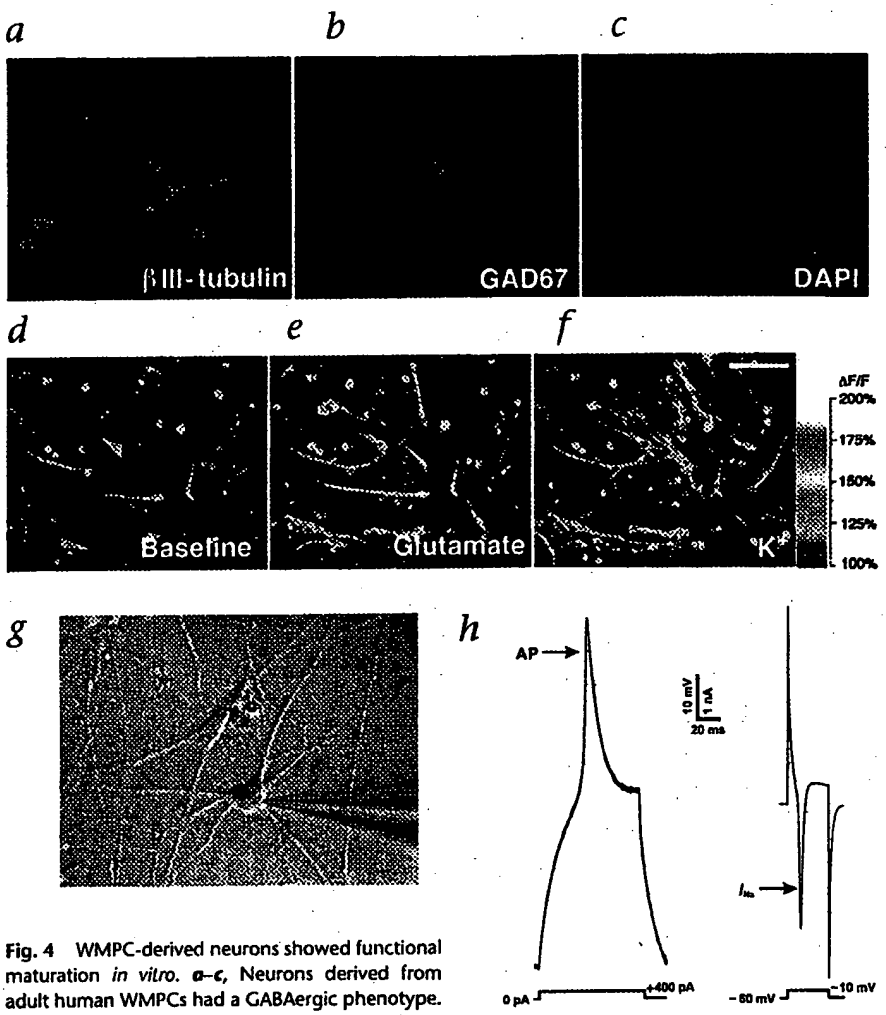

Fig. 4 WMPC-derived neurons showed functional maturation *in vitro*. *a–c*, Neurons derived from adult human WMPCs had a GABAergic phenotype. *a*, Outgrowth of a WMPC-derived neurosphere, stained for neuronal βIII-tubulin after 35 d *in vitro*. *b*, Immunostaining showed that all 9 neurons in the field were GAD67$^+$ and were thus likely to be GABAergic. *c*, DAPI nuclear counterstaining showed the abundance of cells in the field. *d–f*, WMPC-derived neurons developed neuronal Ca^{2+} responses to depolarization. *d*, WMPC-derived cells loaded with the calcium indicator dye Fluo-3, 10 d after plating of first-passage spheres derived from A2B5-sorted white matter (35 d *in vitro* total). Many fiber-bearing cells of both neuronal and glial morphologies are apparent. *e*, The same field after exposure to 100 μM glutamate. *f*, The same field after exposure to a depolarizing stimulus of 60 mM KCl. Rapid, reversible, >100% elevations in cytosolic calcium occurred in response to K^+, consistent with the activity of neuronal voltage-gated calcium channels. Scale bar, 80 μm. *g* and *h*, Whole-cell patch-clamp experiments detected voltage-gated sodium currents and action potentials in WMPC-derived neurons. *g*, Representative cell, 14 d after plating of first-passage sphere derived from A2B5-sorted white matter. The cell was patch clamped in a voltage-clamped configuration and its responses to current injection were recorded. *h*, Action potentials (AP) were noted after positive current injection, at I_{inj} >800 pA (left tracing). The fast negative deflections noted after depolarization steps are typical of the voltage-gated sodium currents of mature neurons (right).

Only a fraction of A2B5$^+$ cells were clonogenic

We next assessed the incidence of clonogenic and multipotential progenitor cells within the larger pool of A2B5-sorted white-matter cells. We first assessed whether either the survival or the mitotic competence of adult human WMPCs were dependent on density, by assessing the limiting dilution at which clonogenic progenitors could be obtained from A2B5-sorted white-matter dissociates. A2B5$^+$ cells were plated immediately after sorting, at densities ranging from 100,000 to 1,000 cells/ml (0.5 ml cell suspension per well of a 24-well plate), in basal media supplemented with bFGF/NT-3/PDGF-AA. Under these conditions, the incidence of clonogenic progenitors was a curvilinear function of the sorted cell density (R^2 = 0.978; Fig. 5*b*). Whereas 186 ± 7.6 spheres were generated at a density of 100,000 cells/ml (0.4%; n = 5 patients), only 6.5 ± 2.7 were noted at 10,000 cells/ml (0.1%) and no sphere generation was noted at or below 5,000 cells/ml. Thus, the expansion of purified WMPCs was density dependent and optimal at 50,000–100,000 cells/ml. Densities higher than the optimal range seemed to promote terminal differentiation of the progenitors.

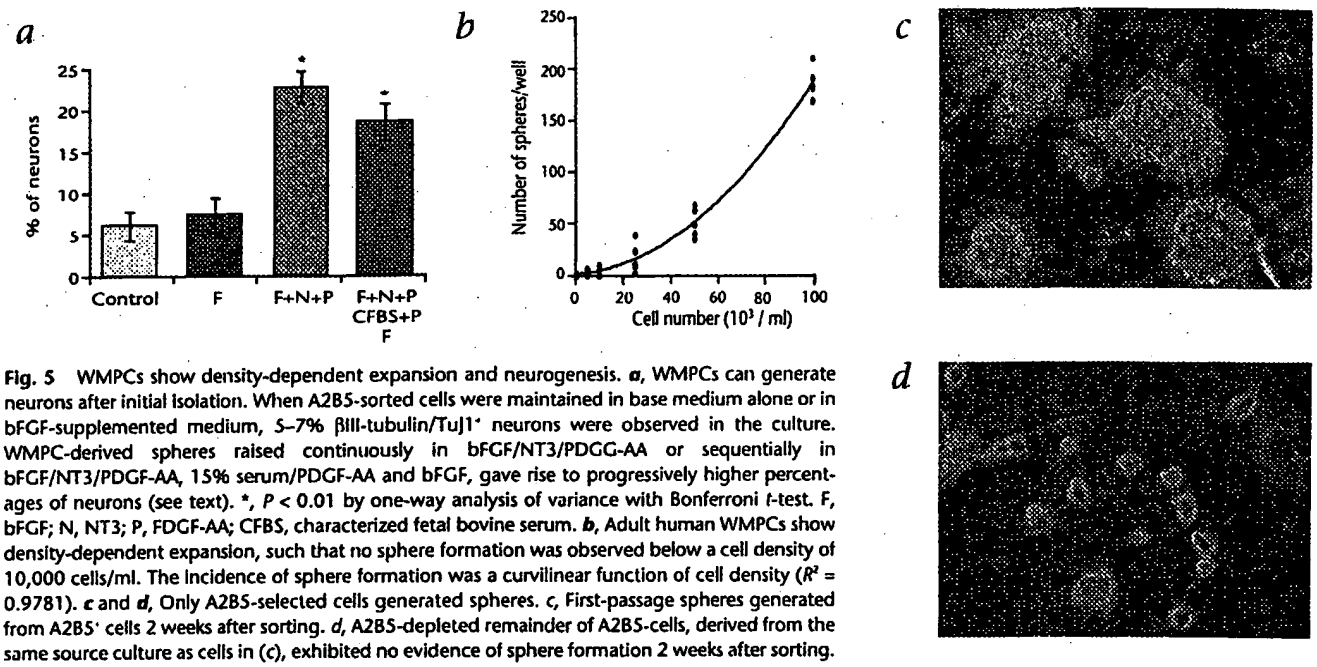

Fig. 5 WMPCs show density-dependent expansion and neurogenesis. ***a***, WMPCs can generate neurons after initial isolation. When A2B5-sorted cells were maintained in base medium alone or in bFGF-supplemented medium, 5–7% βIII-tubulin/TuJ1$^+$ neurons were observed in the culture. WMPC-derived spheres raised continuously in bFGF/NT3/PDGG-AA or sequentially in bFGF/NT3/PDGF-AA, 15% serum/PDGF-AA and bFGF, gave rise to progressively higher percentages of neurons (see text). *, $P < 0.01$ by one-way analysis of variance with Bonferroni *t*-test. F, bFGF; N, NT3; P, FDGF-AA; CFBS, characterized fetal bovine serum. ***b***, Adult human WMPCs show density-dependent expansion, such that no sphere formation was observed below a cell density of 10,000 cells/ml. The incidence of sphere formation was a curvilinear function of cell density (R^2 = 0.9781). ***c*** and ***d***, Only A2B5-selected cells generated spheres. *c*, First-passage spheres generated from A2B5$^+$ cells 2 weeks after sorting. *d*, A2B5-depleted remainder of A2B5-cells, derived from the same source culture as cells in (*c*), exhibited no evidence of sphere formation 2 weeks after sorting.

To assess whether clonogenic WMPCs were restricted to the A2B5$^+$ population, we also cultured the A2B5-depleted pool remaining after each sort. A2B5-depleted cultures did not give rise to any passageable neurospheres at any of the cell densities assessed over the range of 1,000–100,000 cells/ml (Fig. 5*d*). On the basis of these studies, we concluded that only a fraction of white-matter A2B5$^+$ cells are actually clonogenic and multipotential progenitors, although all clonogenic WMPCs are A2B5$^+$.

Adult WMPCs showed limited self-renewal

We next sought to define the extent to which WMPCs were self-renewing by assessing the extent to which WMPC-derived neurospheres were capable of repetitive passage. Primary spheres were raised from three patients at an optimal initial density of 100,000 cells/ml, under the conditions identified as most supportive of multilineage expansion (bFGF/NT3/PDGF-AA in DMEM/F12/N1). One month later, the spheres were dissociated and replated. Secondary spheres were generated and were replated one month later at 1×10^4–5×10^4 cells/ml. These cultures gave rise to tertiary spheres over the following month, though with less efficiency and a smaller volumetric expansion than secondary spheres. Attempts at propagating these spheres as quaternary spheres, after additional dissociation, were generally unsuccessful. Given an apparent cell doubling time of 3–4 d (data not shown) and monthly passages spanning 8–10 doublings, we estimated that the tertiary spheres assessed one month after the last passage underwent a minimum of 16–24 and no more than 30 doublings. This is well below the number of doublings of which tissue-derived stem cells are typically thought capable.

Our inability to successfully passage these cells beyond 16–24 doublings called into question their ability to self-replicate for extended periods of time *in vitro*. Their limited replicative competence contrasted with that of neural progenitors sorted from the fetal human ventricular zone, which may be readily passaged for >60 doublings under analogous culture conditions[21]. Such self-renewal capacity has been ascribed to sustained telomerase activity in a number of developing systems, including the fetal human forebrain[22,23]. To assess whether the apparently finite proliferative potential of adult human WMPCs reflected a lack of telomerase activity, telomerase levels were assessed using the telomerase reverse transcriptase activity protocol (TRAP) assay[23,24]. We did not detect any telomerase activity in primary or secondary WMPC-derived spheres, despite high-level activity in a variety of positive controls (see Supplementary Fig. 1 online). Their lack of extended replicative potential, coupled with their lack of telomerase activity, suggests that adult WMPCs might constitute a pool of multipotential progenitors with a finite capacity for mitotic expansion, transitional between tissue-restricted stem cells and phenotypically committed progenitors.

WMPCs produced neurons and glia after fetal xenograft

We next assessed whether WMPCs were multipotential *in vivo* as well as *in vitro* by evaluating their fate after engraftment to embryonic stage (E)17 fetal rat brains. Some A2B5-sorted cells were transplanted 24–48 h after sorting to assess their lineage potential upon initial isolation. These cells were maintained only in SFM during the period between isolation and xenograft and were never exposed to any exogenous growth factors. Other cells were transplanted 10 d after sorting, after maintenance in bFGF/NT3/PDGF-AA for 4 d and 15% serum/PDGF-AA followed by bFGF, for 3 days each. All donor cells were administered into E17 rat embryos by intraventricular injection at 10^5 cells/animal. The recipients were killed and fixed four weeks after birth to evaluate the fate of the implanted human cells. Human donor cells were identified by immunolabeling of brain sections for human nuclear antigen (HNA).

In rats implanted with propagated WMPCs (Fig. 6) and their counterparts injected with acutely isolated WMPCs (see Supplementary Fig. 2 online), donor-derived migrants co-expressing HNA with either nestin or doublecortin[25] were found in the host olfactory subpendyma and hippocampus (Fig. 6*a* and

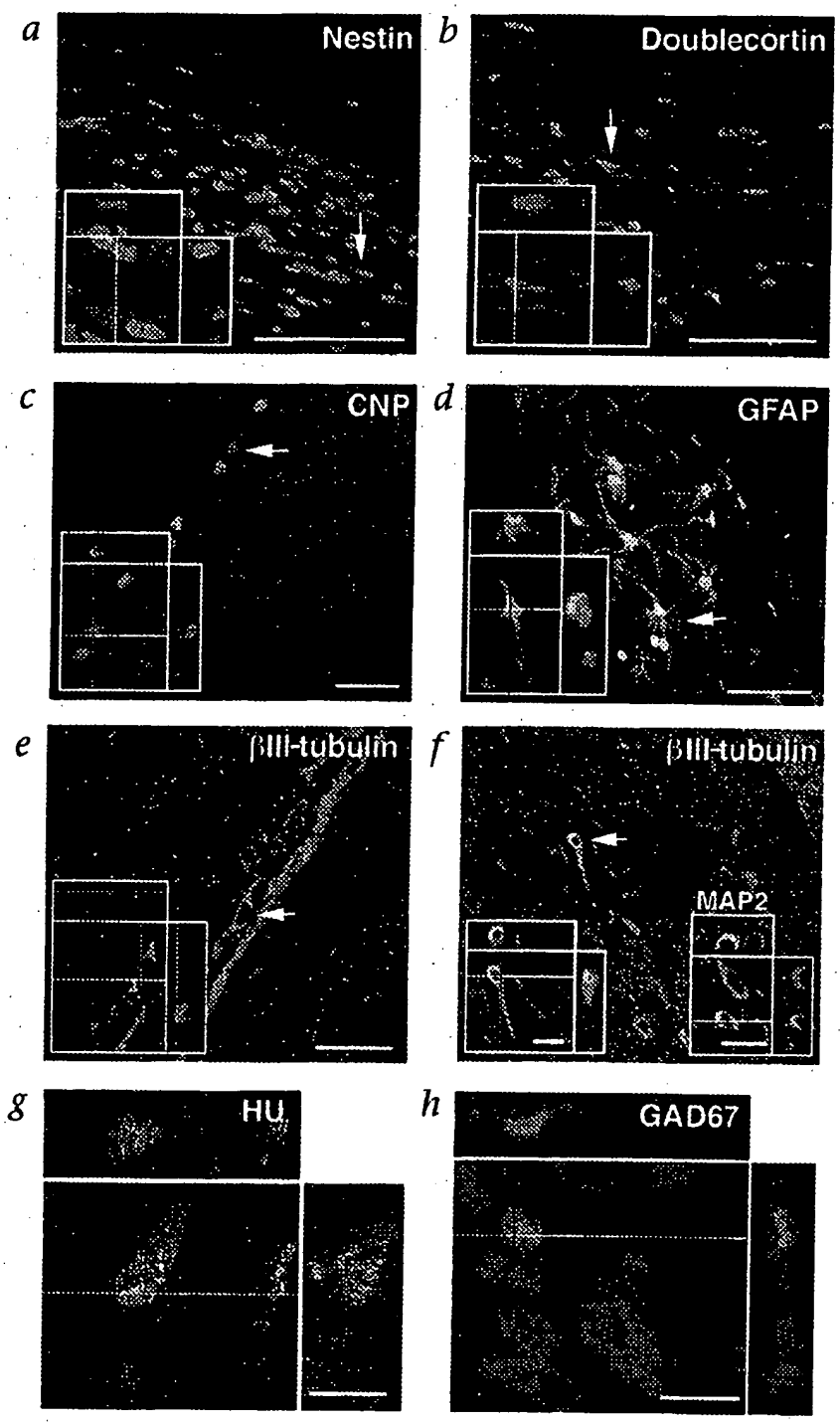

Fig. 6 WMPCs engrafted into fetal rats give rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and killed 1 month after birth. Cells were maintained in culture for 10 d before implanting. ***a*** and ***b***, Nestin⁺ (*a*) progenitors and doublecortin⁺ (*b*) migrants (red) each co-expressing HNA (green) in the hippocampal alvius. ***c***, CNP⁺ (red) HNA⁺ (green) oligodendrocytes, found exclusively in the corpus callosum. ***d***, Low-power image of GFAP⁺ (green) HNA⁺ (red) astrocytes (yellow, double-positive) along the ventricular wall. ***e***, βIII-tubulin⁺ (green) and HNA⁺ (red) neurons migrating in a chain in the hippocampal alvius. ***f***, βIII-tubulin⁺ and MAP2⁺ (inset) neurons in the striatum, adjacent to the rostral migratory stream (green, bIII-tubulin and MAP2; red, HNA; yellow, double-stained human nuclei). ***g***, Hu⁺ (red) HNA⁺ (green) neuron in the septum. ***h***, GAD67⁺ (red) HNA⁺ (green) striatal neuron. Insets (*a–f*) show orthogonal projections of a high-power confocal image of each identified cell (arrow). Scale bars, 40 μm (*a–e*) or 20 μm (*f–h*).

b). In addition, abundant populations of HNA⁺ βIII-tubulin⁺ neurons were found in the olfactory subependyma and rostral migratory stream as well as in the hippocampal alvius (Fig. 6*e*). WMPC-derived neurons were also observed in the neostriatum, indicating striatal neuronal differentiation on the part of some xenografted WMPCs (Fig. 6*f*). These data showed that engrafted adult human WMPCs could integrate into the forebrain subventricular zone as neuronal progenitor cells that then gave rise to both granule and striatal neurons. Human WMPC-derived GFAP⁺ astrocytes and CNP⁺ oligodendrocytes were also common in recipient brains and were found primarily along the ventricles or in the subcortical white matter (Fig. 6*c* and *d*). Thus, adult human WMPCs showed context-dependent differentiation after xenograft to the developing rat brain and were competent to do so upon acute isolation, without the benefit of humoral instruction *in vitro*.

Discussion

These observations suggest that the WMPCs of the adult human forebrain include multipotential progenitor cells, capable of a finite and limited degree of expansion and self-renewal. These cells remain competent to respond to local instructive cues, with a wide range of lineage choices, upon xenograft as well as *in vitro*. They are readily able to give rise to neurons and glia once they are removed from their native white-matter environment. The freshly isolated adult WMPCs in our study did not require prolonged expansion to undergo neurogenesis *in vitro*, and seemed immediately competent to generate neurons upon xenograft to the developing brain.

Previous studies of the adult rat brain have identified parenchymal progenitor cells that are able to give rise to neurons and glia after a number of cell doublings, in the presence of bFGF[8]. In addition, nominally committed glial progenitor cells derived from the neonatal rat optic nerve have also been reported to give rise to neurons and oligodendrocytes[4]. The lineage diversification of these cells seems to require a humorally directed reprogramming of their phenotype, with the induction of an astrocytic intermediary on the way to neurogenesis. In the present study, adult human WMPCs did not seem to require any such reprogramming or transdifferentiation to achieve multilineage competence. Similarly, they did not seem to pass through an intermediate astrocytic stage before generating neurons, oligodendrocytes and astrocytes. Indeed, after their acute isolation and xenograft, A2B5-defined WMPCs were able to

generate all major neural phenotypes *in vivo* and *in vitro*, without any exogenous growth factor exposure. Nevertheless, because an average of 7% of A2B5-sorted white-matter cells co-expressed GFAP (data not shown), it is possible that some WMPCs exhibit astroglial features at some point during their ontogeny, much like subventricular neural progenitor cells[26,27]. This categorization notwithstanding, our results suggest that the WMPCs of the adult human brain are fundamentally tissue-specific progenitor cells that are tonically restricted to glial lineage by the local parenchymal environment, and do not require specific phenotypic reprogramming for neuronal differentiation.

These data suggest that adult human WMPCs constitute a population of parenchymal glial progenitor cells whose *in situ* fate is restricted by the local white-matter environment. Yet the progenitor cell pool of the adult white matter may be heterogeneous, and it is not clear whether all WMPCs have the same ontogeny or fate potential[28–30]. A minority of multipotential progenitor cells might still persist among a larger pool of more fundamentally lineage-restricted glial progenitors[8]. These parenchymal multipotent progenitors may constitute a relatively rare subpopulation, more akin to persistent stem cells than to any lineage-restricted derivatives[31,32]. In this regard, although we did not detect telomerase activity in sorted WMPCs, if the clonogenic portion of these represents only a small fraction of the total progenitor pool, then their numbers might have been below the detection threshold of our TRAP assay. Further study of the heterogeneity of the white-matter progenitor cell population, and of the lineage competence of its constituent phenotypes, will be needed to define the spectrum of progenitor cell types in the adult brain. These considerations aside, multipotential and neurogenic progenitors are abundant in the adult human white matter and are both extractable and expandable. These cells may prove to be important agents for both induction and implantation strategies of cell-based neurological therapy.

Methods

Tissue dissociation and culture. Adult subcortical white matter was surgically obtained from 21 patients, including 14 undergoing epileptic resections (age 1–50 years; 7 males and 7 females), one undergoing aneurysmal repair (69-year-old male), 2 undergoing resections of a noncontiguous dysplastic focus (20-year-old male and 36-year-old female) and 4 undergoing traumatic temporal lobe decompressions (17–67 years old; all males). Samples were obtained from patients who consented to tissue use under protocols approved by the New York Hospital–Cornell and Columbia Presbyterian Hospital Institutional Review Boards. The samples were dissected and dissociated to single-cell suspensions using papain and DNase as described[2,33,34]. The cells were then suspended in DMEM/F12/N1 with either bFGF (20 ng/ml; Sigma, St. Louis, Missouri) alone or bFGF with NT-3 (2 ng/ml; R&D Minneapolis, Minnesota) and PDGF-AA (20 ng/ml; Sigma), and plated in 100-mm suspension culture dishes (Corning, New York).

Magnetic separation of A2B5$^+$ cells. The number of viable cells was determined using calcein (Molecular Probes, Eugene, Oregon) 24–48 h after dissociation. The cells were then washed and incubated with A2B5 supernatant (clone 105; American Type Culture Collection, Manassas, Virginia) for 30–45 min at 4 °C, washed 3 times with PBS containing 0.5% BSA and 2 mM EDTA, and incubated with microbead-tagged mouse-specific rat IgM (1:4; Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C. The A2B5$^+$ cells were washed, resuspended and separated using positive selection columns, type MS$^+$ RS$^+$ or LS$^+$ VS$^+$ (magnetic cell sorting (MACS); Miltenyi Biotech). For flow cytometry of matched samples, cells were incubated in FITC-labeled mouse-specific goat IgM at 1:50 before FACS.

Transfection and sorting. Samples were transfected with pCNP2:hGFP after 2–6 d *in vitro*, using 2 μg of plasmid DNA and 10 μl of Lipofectin (Gibco, Carlsbad, California) as described[2,33,35]. Sorting for pCNP2:hGFP and A2B5 immunofluorescence was performed on a Becton-Dickinson FACS Vantage (San Diego, California), also as described[2,33,35]. Untransfected and IgM-exposed control cells were used to calibrate background; a false-positive rate of 1% was accepted as cutoff.

Generation of primary and secondary spheres. A2B5$^+$ and A2B5-depleted white-matter cells were distributed to a 24-well plate directly after sorting, at 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 cells/ml with 0.5 ml/well of DMEM/F12/N1 with bFGF/NT3/PDGF-AA. The resulting WMPC-derived neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells with trypsin and EDTA. The cells were plated at 3,000 cells/well. Three weeks later, the resultant secondary spheres were either dissociated and passaged again as tertiary spheres, or plated into 2% FBS with 20 ng/ml brain-derived neurotrophic factor on a polyornithine and fibronectin substrate and fixed 2 weeks later.

Lentiviral tagging and lineage analysis. A2B5-sorted cells were infected 2–5 d after separation with lentivirus (10^8 PFU/ml) expressing GFP under CMV promoter control and a WPRE5 post-transcriptional regulatory element[12,13]. The lentivirus was generated by co-transfecting plasmids pCMV/DR8.91, pMD.G, and pHRCMVGFPwsin into 293T cells as described[14]. A2B5-sorted cells were exposed to lentivirus for 24 h in polybrene-supplemented medium (8 μg/ml), then passaged into fresh medium in 24-well plates. GFP expression by tagged cells was observed within 2 d. The primary spheres that arose in these cultures were dissociated 3 weeks later and replated at 3,000 cells/well; secondary spheres arose from these within 2 weeks.

TRAP assay. Telomerase activity was determined using the TRAP assay[23,24], described in detail in the material accompanying Supplementary Figure 1 online.

***In utero* transplantation.** Transuterine xenograft into E17 rat fetuses was performed as described[21,36]. Some cells were injected within 24–48 h after sorting and others after 10 d *in vitro* in FGF2, PDGF-AA and NT3. One month after implantation, the animals were perfusion-fixed by 4% paraformaldehyde. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

Immunocytochemistry. Xenografted rat brains were cryosectioned at 15 μm, permeabilized with PBS, 0.1% saponin and 1% NGS, and blocked with PBS, 0.05% saponin and 5% NGS, each for 30 min. Sections were labeled with HNA-specific mouse antibody (1:50; Chemicon, Temecula, California), then immunostained with βIII-tubulin–specific antibody TuJ1 (1:600; Covance, Princeton, New Jersey), MAP2-specific antibody AP-20 (1:50; Sigma), HuC/HuD-specific mouse monoclonal antibody 16A11 (25 μg/ml; H. Furneaux, Memorial Sloan-Kettering Cancer Center, New York), GAD67-specific rabbit antibody (1:100; Chemicon), GFAP-specific mouse antibody SMI 21 (1:1,000; Sternberger, Lutherville, Maryland), GFAP-specific rabbit antibody (1:400; Sigma), CNP-specific mouse antibody SMI 91 (1:1000 Sternberger), human nestin–specific rabbit antibody (1:200; Chemicon), or doublecortin-specific rabbit antisera (1:100; C. Walsh, Harvard Medical School, Boston, Massachusetts). The sections were incubated with antibody overnight at 4 °C. Species- and isotype-specific fluorescent secondary antibodies were applied at 1:100 for 1.5 h at room temperature.

O4 and A2B5 were immunolabeled *in vitro* as described[2]. For multiple-antigen labeling, O4 was localized on live cells that were then fixed and stained for βIII-tubulin, MAP2, GFAP, Hu, GAD67 or BrdU. O4 supernatant (R. Bansal and S. Pfeiffer, University of Connecticut Health Center, Farmington, Connecticut) was used at 1:100 for 40 min at 4 °C. Antibodies against βIII-tubulin, MAP-2, GFAP and BrdU (BrdU-specific rat antibody; 1:200; Harlan, Indianapolis, Indiana) were incubated overnight at 4 °C. Fixed cultures were counterstained with DAPI (10 μg/ml; Molecular Probes).

Confocal imaging. In the xenografted brains, single cells that appeared co-labeled for both human- and cell-specific markers were evaluated by confocal imaging as described[21,37]. To be deemed double labeled, cells were

required to have HNA-specific signal surrounded by neuronal or glial immunoreactivity in every serially acquired 0.4-μm *z*-dimension optical section, as well as in each orthogonal side view thereof.

Calcium imaging. Outgrowths from both first- and second-passage WMPC-derived neurospheres were assessed 2–3 weeks after plating into BDNF-supplemented DMEM/F12/N1 with 2% FBS. These mixed neuronal and glial outgrowths were challenged with 100 μM glutamate or 60 mM potassium. Cytosolic calcium imaging was conducted using confocal microscopy of cultures loaded with Fluo-3 acetoxymethylester (Molecular Probes)[33,38,39]. We previously reported that adult progenitor–derived human neurons showed a mean calcium rise of >400% in response to 60 mM potassium *in vitro*, compared with glial responses of <20%[38]. In this study, we assigned neuronal identity to cells with ≥2-fold calcium elevations to depolarization.

Electrophysiology. Sister cultures to those subjected to calcium imaging were assessed by whole-cell patch-clamp analysis. Whole-cell voltage-clamped recordings of fiber-bearing cells were conducted and analyzed as described[15,33]. A holding potential of –60 mV and voltage steps of 10 mV with 100-ms durations were applied to the recorded cells through the patch electrodes. Signals were sampled every 50 μs.

Note: Supplementary information is available on the Nature Medicine *website.*

Acknowledgments

This study was supported by US National Institute of Neurological Disorders and Stroke grants R01NS39559, R01NS33106 and R37/R01NS29813, and by the National Multiple Sclerosis Society, Christopher Reeve Paralysis Foundation, and Project ALS. N.S.R. and M.N. were supported in part by the American Heart Association. M.N. was supported by the Fundação para a Ciencia e Tecnologia and the Gulbenkian Foundation of Portugal. We thank D. Trono for the lentiviral plasmids; C. Walsh for anti-doublecortin antisera; H. Furneaux for Hu-specific monoclonal antibody 16A11; R. Bansal and S. Pfeiffer for the monoclonal O4 line; and A. Benraiss, M. Windrem and T. Takano for advice and assistance.

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 4 DECEMBER 2002; ACCEPTED 19 FEBRUARY 2003

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